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كلية الدراسات العليا

التاريـــخ: ٥ / ٤ / ٢٠١١

نموذج رقم (١٨)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية وقوانين الجامعة الأردنية وأنظمتها وتعليماتها لطلبة الماجستير

أنا الطالب: رياض عبد الحميد الزمر الرقم الجامعي: (٨٠٧٠٦٧٣)

تخصص: وقاية النبات الكليـــة: الزراعة

عنوان الرسالة: Detection and identification of Erwinia carotovora pv. atroseptica (van hall, 1902) The causal agent of Potato blackleg by polymerase chain reaction.

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

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DETECTION AND IDENTIFICATION OF *ERWINIA CAROTOVORA* PV. *ATROSEPTICA* (VAN HALL, 1902) THE CAUSAL AGENT OF POTATO BLACKLEG BY POLYMERASE CHAIN REACTION

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Faculty of Graduate Studies
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Committee Decision

This thesis (Detection and identification of *Erwinia carotovora* pv. *atroseptica* (Van Hall, 1902) the causal agent of potato blackleg by polymerase chain reaction) was successfully defend and approved on March. 22.2011.

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DEDICATION

TO MY

PARENTS,

MY

SISTERS AND BROTHERS

AND

TO MY FRIENDS

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bp	Base pair
°C	Degree celsius
cfu	Colony forming unit
DNA	Deoxyribonuclic acid
dNTPs	Deoxynucleoside triphosphate(s)
Eca	*Erwinia carotovora* pv. *atroseptica*
Ecc	*Erwinia carotovora* pv. *carotovora*
ELISA	Enzyme-linked immunosorbent assay
h	Hour(s)
min	Minute(s)
µM	Micromolar (s)
mM	Millimolar(s)
NA	Nutrient agar
PCR	Polymerase chain reaction
%	Percent
rpm	Round per minute
RFLP	Restriction fragment length polymorphism
Taq	*Thermus aquaticus*
TBE	Tris Borate- ethylenediamine tetra acetic acid
U	Units of enzyme
UV	Ultra violet
V	Voltage
v	Volume
w	Weight
w/v	Weight/ volume

UPGMA	Unweighted pair-group method using arithmetic average

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ABSTRACT

The results of this study showed that potato blackleg disease was recorded to spread in different potato growing areas in Jordan including; Jordan Valley (AlKarama& Ash shuna janubiyya, Muthallath al misri, Mu'addi, Dayr Alla, Dirar, and Kurayyima), Amman (Al yadoda), Madaba (Omalamad), Jarash (Tawahin Al udwan), Ma'an (Ram, and Al mudawwara).

Forty six bacterial isolates of *Erwinia carotovora pv. atroseptica* (*Eca*) the causal agent of bacterial blackleg of potato, were isolated and identified by different biochemical, physiological and nutritional tests from 145 diseased potato samples collected from the different potato growing areas in Jordan and the results of the reaction were found identical to the *Eca* reference culture (ATCC 15713).

PCR amplification of a 690-bp band was used to confirm the presence of *Eca* in the isolated bacteria and reference culture (ATCC 15713).

PCR amplification was compared in regard to direct isolation and identification to detection of *Eca* in infected and symptomless potato stems and tubers. The results showed that *Eca* was isolated from 30% of naturally infected potato samples with blackleg, while

the 690-bp band was detected in 88% of DNA extract amplified by PCR of the naturally infected potato samples. In the other hand *Eca* was not isolated from the symptomless potato stem samples, while the 690-bp band was detected in 8% of the DNA extract of these stem samples amplified with PCR. However; using symptomless tuber samples has improved isolation efficiency, Eca was isolated directly from 4% of the samples when their suspension was streaked on Logan's media plates and the 690-bp band was detected from 32% of their DNA extract amplified with PCR.

For the detection of molecular variation among the *Eca* isolates, the 690-bp PCR products of the 46 different isolates were digested with the restriction enzymes *Alu*I, *Hae*II, *Hpa*II and *Sau*AI and the PCR-RFLP analyses divided the *Eca* isolates to 5 PCR-RFLP groups with low genetic variability.

1. Introduction.

Potato (*Solanum tuberosum* L.) is considered an economic crop in many countries in the world including Jordan. Potato ranks the second among the vegetable crops grown in Jordan, where the area planted with potato in 2008 was 56700 dunums with total production of 189100 tons. Of this area, 54.3 % was planted in the Jordan Valley (Annual Agricultural Statistics, 2008).

Different bacterial diseases have been reported world wide to attack potatoes of these: soft rot (*Erwinia carotovora* subsp *carotovora* (Jones)), brown rot (*Ralstonia solanacearum* (Smith 1896)), ring rot (*Clavibacter michiganensis* subsp *sepedonicus* (Spieckermann & Kotthoff)), potato scab (*Streptomyces scabies* (Waksman & Henrici 1943) (Stead, 1999)). Blackleg (*Erwinia carotovora* subsp *atroseptica* (Van Hall, 1902) Dye 1969).

Blackleg disease is mainly restricted to temperate climates, and it is one of the most important diseases attacking potato and drastically affects its yield resulting in economic losses (Elphistone, 1987; Cappaert *et al.*, 1988, Bell *et al*., 2004). In USA, Kennedy and Alcorn (1980) estimated the annual losses in potatoes, due to blackleg disease, to be around 14 million dollars.

Blackleg has a restricted host range, where potato being the major host plant in cold to temperate regions (Perombelon and Kelman, 1987; George *et al.*, 1991, Helias et al., 1998; Helias *et al.*, 2000), Sugar beet and sunflower were also reported as other hosts (Stanghellini *et al.*, 1977; Mendonga and Stanghellini, 1979; Whiteney, 1982; Gudmestad *et al.,* 1984; Jorge and Harrison, 1986; Schuerger and Bartz, 1993).

Eca is a seed born pathogen where contaminated tubers, diseased or infected propagative materials are considered as primary inoculums sources for blackleg disease (Ward and DeBoer, 1994; Van der Wolf *et al.*, 1996; Al-Masa'deh and Khlaif, 2003).

Because blackleg is a seed born pathogen, it is one of the potato diseases under quarantine in Jordan. To restrict the spreading of the disease, different methods were followed to detect the pathogen from infected or contaminated potato tubers or latent infection, with varying degrees of specificity, sensitivity and suitability for large scale use (Perombelon and Hyman, 1995). Of these methods direct isolation on common and selective media, such as crystal violet pectate (CVP), where the isolation of the causal agent could be seriously affected by the presence of large numbers of saprophytic bacteria (Jones *et al.*, 1994). Immunological methods, such as Enzyme-linked immunosorbent assay (ELISA), was found to be not efficient in detecting low population of the pathogen and can't detect all *Eca* different strains (De Boer *et al.*, 1987; Gorris *et al.*, 1994). However these methods are not highly efficient in detecting the low population of the pathogen, time consuming, and expensive. Therefore a rapid and efficient diagnostic technique for detection and identification of *Eca* is needed. Recently Polymerase chain reaction (PCR) proved to be highly sensitive and specific method for the detection of blackleg pathogen in potatoes (De boer and Ward, 1995, Perombelon and Hyman, 1995).

This research was conducted in order to:

1- Isolate and identify *Eca*, the causal agent of potato blackleg disease, from different potato growing areas in Jordan Valley and Uplands.
2- Detect and identify *Eca* from the obtained bacterial isolates, diseased and healthy tubers, and plants samples by PCR technique.

3- Check if there is genetic diversity among the different obtained bacterial isolates through PCR- restriction fragment length polymorphism.

2. Literature review.

Erwinia carotovora subsp. *atroseptica* (*Eca*) is the causal agent of potato blackleg disease. It is a gram-negative, nonspor forming, peritrichous flagellate, facultative anaerobic bacteria, characterized by the production of large quantities of extracellular pectic enzymes (Perombelon, 2002).

Bacterial blackleg is a seed-born pathogen occurs in all potato (*Solanum tuberosum* L.) growing areas around the world **(**Fredricks and Metcalf, 1970; Powelson, 1980; Powelson and Apple, 1986 Cappaert *et. al.*, 1988). In Jordan, Blackleg disease was reported to occur in the Jordan Valley as well as in the Uplands with various degrees (Al-Masa'deh and Khlaif, 2003).

Blackleg has been recognized since the turn of the century as a disease capable of causing tremendous losses to the crop in the field or during storage (Fredricks and Metcalf, 1970). The economic importance of blackleg is twofold: it can reduce the yield, especially when incidence exceeds 5-10% and can cause downgrading or rejection of seed-potato crops submitted for certification (Perombelon, 2000). *Eca* is able to cause symptoms both during the vegetative cycle of the crop and on stored tubers, and is therefore considered the most threatening disease for potato production (Lewosz, 1992).

2.1. Host range and Symptoms

Blackleg has a restricted host range, where potato being the major host plant in temperate regions (George *et al*., 1991). Infected potato plants may appear healthy without external symptoms as latent infection, but under wet conditions, the bacteria becomes active in the xylem vessels penetrate and macerate the adjacent parenchymatous tissues, resulting in yellowing and wilting of leaves and stunting of some or all stems. Leaflets often show curled margins and wilting symptoms are strongest at midday. In later stages whole stems and plants will wilt completely and collapse. When stems are pulled out of the ground they show a typical black discoloration and have a typical bad smell of rotting fish. The vascular tissue may be brown in color. Infected tubers show a brown to black rot that is glassy, but not soft, like the soft rot caused by *E. c.* subsp. *carotovora*. The infection usually starts at the heel end and can also be observed on the outside of the tuber as sunken purple to black zones around the heel end (Janse, 2005).

Potato blackleg symptoms can either be a stem or plant wilting and desiccation, resulting from absence of water flow caused by bacteria and gum-like material and tyloses which originate from the point of attachment to the mother tuber causing weakened plants, premature death, and loss of stands (Powelson, 1980; Helias *et al.,* 2000; Perombelon, 2000).

2.2. Source of inoculums and optimum condition for infection

Eca is a seed-born pathogen and the contaminated mother tubers were reported as the main source of inoculums for the disease (Ward and DeBoer, 1994), the bacteria can survive in a quiescent form in lenticels and wounds caused by insect larvae, fertilizer burns, frost injury, bruises, or lesions caused by other pathogenic bacteria and fungi (Fredricks and Metcalf, 1970). Other sources of inoculums are airborne (insects and aerosols) where are *Eca* deposited on

leaves and from there to the soil and progeny tubers, and *Eca* in rotting tubers smeared into wounds incurred during mechanical crop handling (Perombelon, 1992). Temperature and soil humidity were reported of the main factors affecting pathogenecity (DeBoer and McNaughton, 1987) and the optimum temperature for potato plants and tuber infection was reported to be around 20°C (Costa *et al.*, 2006). In Jordan, Al-Masa'deh and Khlaif, (2003) reported that the contaminated tubers and diseased plant debris are the major source of inoculums for blackleg disease. They also reported that the maximum diseases development occurred in the Jordan Valley was found to be coincided with an average soil temperature of 16.3 °C, an average air temperature of 16.4 °C, an average soil moisture of 16.5 %, and an average relative humidity of 72.3 %. Higher percentage of potato yield losses due to blackleg were reported when potatoes wintering planted (Al-Masa'deh and Khlaif, 2003).

In Japan the intensity of blackleg symptoms at 18 °C was higher than that at 24.5 °C (Tani *et al.* 1973). Bain et al. (1990) found a clear relationship between the number of *Eca* bacteria present on potato tubers and the incidence of blackleg in the field. Moreover, the incidence of blackleg late in the season was negatively correlated to tuber yield.

2.3. Disease control

Control of this disease is based on planting clean seed identified by quantifying tuber contamination rather than visual inspection, sanitation, crop rotation, planting in well-drained soils, prevention of any wounds or diseases which could permit entry of the pathogen, planting low susceptible cultivars and proper storage conditions for freshly dug tubers and cut tubers if they cannot be planted immediately (Fredricks and Metcalf, 1970; Perombelon, 1992).

In temperate regions much attention has been paid to improve the quality and health of seed potatoes. Field inspection for blackleg failed to assure the status of this pathogen in seed

lots, because post-harvest contamination and latent infections cannot be detected by visual observation (Fraaije *et al*., 1997).

2.4. Detection of *Eca*

The available techniques for detection and identification of *Eca* were based on microscopy, isolation, biochemical characterization, serological techniques, bioassay and pathogenicity tests (Lopez *et al.*, 2003).

Techniques based on dilution plating, serology and DNA technology have also been developed to detect *Eca* on potato tubers (Fraaije *et al.*, 1997). Dilution plating on crystal violate pectate (CVP) medium lack sensitivity due to the presence of large numbers of potato tuber-associated saprophytes and *Erwinia carotovora* subsp *carotovora* (*Ecc*), however both are unreliable and time-consuming (Janse and Spit, 1989).

Immunofluorescence cell and colony staining (Allan and Kelman, 1977), although laborious, and ELISA, in combination with an enrichment step (Gorris *et al.*, 1994), have good potential for routine application. However, serological techniques sometimes show false positive and negative reaction, due to cross-reacting with saprophytes (Van der Wolf *et al.*, 1996) and variation in serotypes (De Boer and McNaughton, 1987). The above mentioned methods and techniques lack sensitivity and can't detect low populations less than 10^2 -10^4 colony forming units (cfu/ml) of *Eca* which is the minimum amount of inoculums needed to incite blackleg symptoms under most field condition (Bain *et al.*, 1990).

Polymerase chain reaction (PCR) assay is widely used as a sensitive and effective technique for the detection of latent infection of *Eca* in potato tubers. The nucleotide sequence for a DNA hybridization probe specific for *Erwinia carotovora* subsp. *atroseptica* was determined, and primers were selected for detection of the blackleg pathogen using (PCR).

ECA1f (5'-CGGCATCATAAAAACACG-3') and ECA2r (5'-GCACACTTCATCCAGCGA-3') primers specifically amplified a 690-bp DNA fragment of all *E. carotovora* subsp. *atroseptica* strains but not strains of other *E. carotovora* subspecies. To compare between the PCR and ELISA, 170 samples of potato stem and tuber tissue were tested by PCR and compared with reactions in ELISA (De Boer and Ward, 1995). In this study, 50.6% of these samples were positive in PCR compared to 46.5% in ELISA. Darrasse *et al.*, (1994) used the primers Y45 (5'-TCACCGGACGCCGAACTGTGGGGT-3') and Y46 (5'-TCGCCAACGTTCAGCAGAACA AGT-3'), derived from a DNA probe specific to *E. carotovora* subsp. *atroseptica* in which they amplify a 439-bp DNA fragment. In 1998 Frechon *et al.* compared PCR test by using the Y45 and Y46 primers with other *Eca* detection methods, using naturally contaminated tubers. Results matched most closely those from viable counts on a selective medium, the most sensitive method (88%), followed by enrichment ELISA (72%) and last ELISA (30%), the sensitive method.

2.5. Detection of possible genetic diversity among *Erwinia carotovora* subsp. *atroseptica.*

Different methods were reported to detect the diversity among *Eca* strains. The purposes of diversity studies were to investigate possible relationships between molecular diversity, geographical origins, and to quantify the relative virulence of the isolates to separate between highly virulent and nonvirulent strains (Helias *et. al.,* 1998, Yap *et. al*., 2004).

According to biochemical tests, Vantomme *et. al.*, (1985) found that *Eca* strains were consisted of two biochemically different groups. De Boer, and Sasser (1987), a result of serological studies, showed that *Ecc* was more diverse than *Eca*, since *Eca* isolates were members of only 4 of the 40 serogroups (serogroups 1, 18, 20, and 22), while *Eca* isolates were members of 38 of the 40 serogroups (serogroups 2 to 21 and 23 to 40).

Yabiaoui-Zaidi *et al*. (2003) used restricted fragment length polymorphism (RFLP) fingerprinting to examine genetic diversity in 14 *Eca* isolates. Restriction profiles, showed that all of the 434-bp PCR product obtained with primers Y1 and Y2 in each of the 14 *Eca* isolates and digested with *Alu*I, *Hae*III, *Hpa*II and *Sau*3AI restriction endonuclease enzymes *Eca* isolates belong to single RFLP group.

Variation in heterogeneity or homogeneity were reported to occur between *Eca* strains, however RFLP analyses for 32 *Eca* isolates by digesting the 434-bp amplified fragments with several restriction enzymes were divided into two subgroups (Darrasse *et al*., at 1994).

In another study (Helias *et al*., 1998), 28 *Eca* isolates were found to belong to two RFLP groups when 434-bp amplification products were digested with *Alu*I, HaeIII, HpaII and *Sau*3AI restriction endonuclease enzymes. 13 isolates of *Eca* occupied 2 different RFLP groups and 57 isolates of *Ecc* occupied 18 different RFLP groups when the 730-bp (recA) amplified DNA fragments were digested with four endonucleases *Alu*I, *Hin*fI, *Tas*I and *Tru*II (Waleron *et al*., at 2002).

3. Materials and Methods:

3.1. Samples collection:

Potato samples including tubers and stems suspected to be infected with blackleg were collected from different potato growing areas in Jordan including: Jordan Valley (AlKarama & Ash shuna janubiyya, Muthallath al misri, Mu'addi, Dayr Alla, Dirar, Kurayyima), Amman (Al yadoda), Madaba (Omalamad), Jarash (Tawahin Al udwan), Ma'an (Ram, Al mudawwara), the collected samples were placed in an ice box and brought to the laboratory for further work.

3.2. Disease incidence for plants:

The disease incidence of the infected plants was calculated in the field during sample collection as a percentage of infected plants out of 100 plants taken randomly in the inspected fields (Al-Masa'deh and Khlaif, 2004).

3.3. Isolation of the causal agent:

The infected tubers or stem samples were placed in washing jars under tap water to remove adhering soil particles; surface disinfected with sodium hypochlorite 0.5 % for 3 minutes, rinsed three times with sterile distilled water and then plotted to dry onto sterile filter paper. A sample of 10 g of plant tissue were aseptically separated with sterile scalpel, cut into small pieces, soaked in 90 ml of sterile distilled water in a sterile milk dilution bottle and placed onto a shaker at room temperature at 200 rpm till the suspension become turbid and homogenized. Then 10 fold dilutions were prepared from the suspension up to the 10^{-4} , 0.1 ml from 10^{-3} and10^{-4} were separately spread by sterile glass rod onto the surface of dried Logan's medium plates (Nutrient agar 24g, yeast extract 5g, glucose 5g and after autoclaving, 10 ml of filter sterilized 0.5% of 2, 3, 5-triphenyl tetrazolium chloride were added). The inoculated

plates were incubated at 25 ±2 º, checked periodically for the growth of colorless colonies within the first 24 hrs of inoculation suspected to be *Eca*, single colonies were re-streaked onto new Nutrient Agar (NA) plates. The obtained bacterial isolates were grown on NA's slants and placed in refrigerator for further identification (Schaad, *et al.*, 2001).

3.4. Identification of the causal agent:

Twenty four hours old cultures of the obtained bacterial isolates were biochemically and physiologically characterized and identified as described by (Schaad *et al.,* 2001, Perombelon and Vander wolf, 2002). The same tests were run against a reference culture of *Eca* isolate (ATCC 15713) obtained from Phytopathogenic bacteria Laboratory, Department of Plant Protection, Faculty of Agriculture at the University of Jordan, Amman, Jordan. These tests included:

3.4.1. Oxidase test:

The purpose of this test is to detect the presence of cytochrom C protein in the tested bacteria. A loop-full of the tested bacterial growth was spotted on a sterile filter paper saturated with freshly prepared 1% (w/v) aqueous solution of tetramethyl-paraphenyl endeiamine dihydrochloride placed into sterile Petri-dish. The observation of dark blue color within (10-60) seconds was considered as a positive reaction (Schaad *et al.,* 2001).

3.4.2. Catalase test:

The purpose of this test is to detect the presence of catalase enzyme. A loop-full of the bacterial culture was placed into a drop of 3% aqueous solution of hydrogen peroxide (H_2O_2) on a sterile microscopic slide. Bubbles formation indicated that the reaction was a positive one (Schaad *et al.*, 2001).

3.4.3. Potato soft rot:

The purpose of this test is to detect the pectolytic activity of the tested bacterium. A v-shaped groove was cut into a potato slice by using sterile scalpel. A loop-full of each tested bacterial growth was separately placed into the groove then inoculated slices were incubated at 27 ± 2 °C in a moist chamber for 24-48 hours. Rotting of the potato slices was considered as a positive result (Schaad *et al.,* 2001).

3.4.4. Oxidative fermentative (OF) test:

This test is performed to detect the ability of the bacteria to utilize glucose oxidatively or fermentatively. A loop-full of the tested bacterial culture was stab inoculated into a test tube of a semi-solid basal medium (peptone 2 g, NaCl 5 g, diapotassium phosphate (K_2HPO_4) 0.3 g, bromothymol blue0.03 g, Agar 3 g and 1.0 % dextrose were added to 1 liter distilled water), inoculated tubes were covered with a layer of sterile 2.0 % molten agar and incubated at 27 ± 2 °C for 24-48 hrs. Changing of the color from dark green to yellow was considered as a positive reaction (Schaad *et al.*, 2001).

3.4.5. Growth at 37°C:

The purpose of this test is to detect the effect of temperature on the growth of the tested bacteria. A loop-full of the bacterial culture was streaked onto the surface of a dried NA plates. Then the inoculated plates were incubated at 37± 2°C for 48-72 hrs. Developing bacterial growth onto the surface of the inoculated plates was considered as a positive result (Schadd *et al.,* 2001).

3.4.6. Sodium chloride tolerance test:

The purpose of this test is to detect the ability of the bacteria to grow on 5% NaCl. A loop-full of the tested bacterial growth was streaked onto the surface of a dried NA plate supplemented with 5% NaCl (w/v). Then the inoculated plates were incubated at 27 ±2°C for 24-48 hrs. Developing of bacterial growth onto the surface of inoculated plate was considered as a positive reaction (Schaad *et al*., 2001).

3.4.7. Starch hydrolysis test:

The objective of this test is to detect the presence of amylase enzyme. A loop-full of the tested bacteria was spot inoculated onto the surface of a dried NA plates supplemented with 0.2% potato soluble starch. Inoculated plates were incubated at 27± 2 °C for 48-72 hrs. And then the plates were flooded with lugol's iodine (Iodine 5 g, potassium iodide 10 g into 100 ml distilled water). Formation of clear zone around the bacterial colony indicated a positive reaction (Lelliot and Stead, 1987).

3.4.8. Nitrate reduction test:

This test is used to detect the ability of the bacteria to produce nitrite from nitrate in gaseous form and utilize O_2 in respiration. A loop-full of the tested bacterial growth was stab inoculated into test tubes containing semi-solid nitrate medium (KNO_3 1g, peptone 5g, Yeast extract 3g, and 3g of agar were added to 1 liter of distilled water). Inoculated tubes were incubated at 27± 2 °C for 7 days. Tubes were checked periodically for bubbles formation as an indication of gas production, which was considered as a positive reaction (Lelliot and Stead, 1987).

3.4.9. H_2S production test:

The purpose of this test is to detect the ability of the tested bacteria to produce H_2S from cystine hydrochloride. A loop-full of the bacterial growth was stab inoculated into a test tube containing H_2S broth medium (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 5g NaCl, 5g of yeast extract, and 0.1 g of cystine hydrochloride added to one liter distilled water, then dispensed into 5 ml test tubes). Sterilized filter paper stripes moistened with 10% of neutral lead acetate solution placed into test tubes 5 mm above the surface of the liquid medium. Inoculated tubes were incubated at 25 ± 2 °C up to 14 days. Blackening of the strips indicated the production of H_2S and was considered as a positive result (Dye, 1969).

3.4.10. Indole production test:

This test is used to detect the ability of the tested bacteria to produce indole from tryptophane. Test tubes containing indole broth medium (10 g of tryptone, 1 g of L-tryptophane in 1 liter of distilled water) were inoculated with a loop-full of bacterial growth. Inoculated tubes were placed onto a shaker at 25 ± 2 °C at 200 rpm for 48 hrs, then 0.5 ml of indole Kovacks reagents (75 ml of amyl alcohole, 25 ml of HCl, and 5 g of P-dimethyl benzyl aldehyde) was added to 5 ml of the broth medium. Tubes were then gently shaken. Changing the color from bright yellow to light pink was considered as a positive reaction (Gerhardt, 1981).

3.4.11. Urease production test:

The purpose of this test is to detect the presence of urease enzyme. A loop-full of bacterial growth was stab inoculated into a test tube containing urease broth medium (0.5 g $NH_4H_2PO_4$, 0.5 g K_2HPO_4, 0.2 $Mg_2SO_4.7H_2O$, 5 g NaCl, 5 g yeast extract and 0.016 g creasol red were added to 800 ml sterile distilled water; after media autoclaving 10% filter sterilize

urea solution was added to the medium to complete up to 1 liter). The medium was dispensed into 5 ml sterile test tubes, and then the inoculated test tubes were placed onto incubator shaker at 27 ± 2 °C and 200 rpm for 48 hrs. Changing the color from pink to dark red was considered as a positive reaction (Schaad *et al*., 2001).

3.4.12. Reducing substances from sucrose:

The purpose of this test is to detect the presence of sucrase enzyme. The test medium contained: 10 g peptone, 5 g beef extract, 40 g sucrose, and 1l distilled water. Then a loop-full of the tested bacteria was inoculated into tubes containing the sterilized medium and placed on shaker-incubator at 27 °C for 2 days. Equal volumes (5 ml) from culture medium was added to 5 ml of Benedict's reagent (173 g sodium citrate and 100 g $Na_2CO_3.H_2O$ were added to 800 ml distilled water, then the suspension was heated while stirring and then filter sterilized under vacuum. After that water was added to bring volume to 850 ml. while stirring 17.3 g $CuSO_4.5$ H_2O was added to increase volume to 1,000 ml. Stored in brown bottle) in a clean test tube and placed in boiling water bath for 10 min. the production of yellow-orange to brown color (with or without precipitate) constituted a positive reaction.

3.4.13. Acid production from α-methyl-d-glucoside:

The purpose of this test is to detect the ability of the tested bacteria to produce acid from α-methyl-d-glucoside. The tested medium contained (2X salts (KH_2PO_4 0.4 %, K_2HPO_4 1.4 %, NH_4Cl 0.2 %), 2X agar (Bacto-agar 3.0 %, Casamino acid 0.2 %), 10 % $MgSO_4$. $7H_2O$, 20 % α-methyl glucoside, 1 % tetrazolium chloride). After Autoclaving these solutions, a mix of 500 ml from each of the first two solutions was prepared (to give a final volume of 1 liter). Then 1 ml/ liter. 10% $MgSO_4$. $7H_2O$, 50 ml / liter of 20 % α-methyl glucoside, and 2 ml/ liter of 1 % tetrazolium chloride were added to the previous mixture. Then the resulted mixture poured

into Petri dishes. Then a mass of the tested bacterial growth was spot inoculated on the plates and incubated at 20 °C for 48 h. The presence of red colony was considered as a positive.

3.4.14. Hypersensitive reaction to tobacco:

The purpose of this test is to separate pathogenic Erwinia isolates from saprophytic ones. The test is performed as follow: Tobacco (white burley) leaves were injected with 10^8 CFU/ml of the tested bacterial suspension by a 3 ml sterile disposable syringe in the lower surface of the tobacco leaves prepared from 24 hrs old culture adjusted to 10^8 CFU/ml using spectrophotometer. Complete collapse of the inoculated tissue and necrosis formation within 24 hrs without developing water-soaked spots was considered a positive reaction (Klement, 1964).

3.5. DNA Extraction from the bacterial cultures:

Erwinia carotovora subsp *atroseptica* isolates were grown on NA plates at 25°C for 24 hrs, then from each isolate few bacterial colonies were picked with a sterile loop and mixed thoroughly in 500 µl sterile ultra pure water and then heated to 50 °C for 3 hrs. DNA was precipitated from the supernatant fractions by adding 1 volume of isopropanol and then pelleted by centrifugation at 15,000 rpm for 4 min. Pellets were washed with 70 % ethanol, dried at 58 °C for 10 min, then re-suspended in 100 µl of nuclease-free water (Promega), and stored at -20 °C for further PCR work (De Boer and Ward, 1995, Helias *et al*., 1998).

3.6. Bacterial DNA Extraction from plant samples (infected and symptomless plant material):

Plant tissues from stems and tubers were macerated with sterile distilled water by using mortar and pestle, then stored on ice for 10 min to allow debris and starch to settle. Then the

supernatant was taken to new centrifuge tube and centrifuged at 5000 rpm to remove excess plant debris. After that the supernatant was pour off into new centrifuge tube and centerfuged at 14000 rpm to pellet the bacterial cells. Then the supernatant was discarded without disturbing the pellet and washed with 70 % ethanol, dried at 58 °C for 10 min, then re-suspended in 100 µl of nuclease-free water (Promega), and stored at -20 °C (De Boer and Ward, 1995, Helias *et al*., 1998, Smid *et al*., 1995).

3.7. PCR amplification:

In order to detect the presence of the 690-bp DNA fragment that confirmed the presence of *Eca*, ECA 1f forward primer (5'-CGGCATCATAAAAACACG-3') and ECA 2r reverse primer (5'-GCACACTTCATCCAGCGA-3') were used (De Boer and Ward, 1995). The PCR reaction mix contained 5 µl (10x) Taq DNA polymerase buffer, 4 µl (25 mM) $MgCl_2$, 0.5 µl of (10mM) dNTPs mixture, 0.3 µl of (50 µM) of each primers, 0.5 µl (1/10) of Tween 20, 0.2 µl of Taq DNA polymerase, 2 µl of template DNA and ultra-pure water to a total volume of 50 µl. Amplification reaction was performed in a thermal cycler (9700 Perkin-Elmer) using the following protocol: Initial denaturation at 95 ºC for 5 min, followed by 36 cycles of (30 s at 94 °C) for denaturation, (45 s at 62 °C) for annealing, and (45 s at 72 °C) for extension and an additional extension step at 72 °C for 8 min. After amplification reaction 10 µl of the PCR product mixed with 2 µl of bromophenol blue as loading dye (6x) were separated by 1.2 % (w/v) agarose (Promega) electrophoresis in 1x TBE (Tris Borate EDTA) buffer. Ethidium bromide of 1 µl per 10 ml of agarose gel was used as detection agent and the 50 and 100-bp ladder were used as a molecular marker. Electrophoresis were performed at 100 V for 1 hrs. Then gels were photographed under UV light (302 nm).

3.8. PCR-RFLP analysis:

The DNA from PCR product for each *Eca* isolate (690-bp DNA fragment) was digested separately with 4 different restriction endonuclease enzymes *Alu*I, *Hae*III, *Hpa*II and *Sau3A*I at 37 °C for 2 hrs as incubation period. The total volume of the digestion reaction for each enzyme was 20 µl, containing 8 µl from PCR product, 10 µl ultra-pure water and 1 U of the enzyme diluted in 2 µl of the appropriate buffer. The digested products were then separated by electrophoresis (100 V, 1 h) on a 2.4 % (w/v) agarose gel Then photographed under UV light (302 nm) as described by Yahiaoui-Zaidi *et al.*, 2003.

3.9. Data analysis:

Amplification products (bands) for the 46 bacterial isolates were scored as 1 for presence, 0 for absence and 9 for missing bands. Band size was identified in base pair. Similarities between isolates were estimated according to Dice (1945). Cluster analysis was conducted using the unweighted pair-group method using arithmetic average (UPGMA). All genetic analyses were conducted using in NTSYSpc 2.0 software (Rohlf, 1998).

4. Results:

4.1. Samples collection:

One hundred forty five diseased potato samples suspected to be infected with blackleg (Fig. 1) were collected during February to April, from different locations in Jordan including: Jordan Valley (AlKarama& Ash shuna janubiyya, Muthallath al misri, Mu'addi, Dayr Alla, Dirar, and Kurayyima), Amman (Al yadoda), Madaba (Omalamad), Jarash (Tawahin Al udwan), Ma'an (Ram, and Al mudawwara) (Table 1).

4.2. Disease incidence

The recorded disease incidence at the time of samples collection varied between the different locations and ranged from 5% to 28% for Madaba and Al mudawwara location respectively (Table 1).

4.3. Isolation of the causal agent:

When the suspension of the natural diseased potato samples were streaked onto the surface of dried Logan's plates small, circular, colorless bacterial colonies were developed after 24 hours (Fig. 2).

Table (1): Locations, sampling date, disease Incidence, number of collected samples and the obtained isolates of *Erwinia carotovora* subsp. *atroseptica*.

Location	**Sampling date**	**Incidence of disease at collection time**	**No. of collected samples**	**No. of Obtained *Eca* isolates**	**Isolates percentage from total isolates**
Jordan Valley					
AlKarama & Ash shuna janubiyya	4.3.2010	16%	8	2	4.3%
Muthallath al misri	22.2.2010	26%	23	9	19.6%
Mu'addi	20.2.2010	14%	10	_	-
Dayr Alla	7.3.2010	22%	20	4	8.7%
Dirar	23.2.2010	16%	8	2	4.3%
Kurayyima	8.3.2010	10%	5	3	6.5%
Amman					
Al yadoda	10.4.2010	20%	8	_	-
Madaba					
Omalamad	10.4.2010	5%	5	_	-
Jarash					
Tawahin Al udwan	6.4.2010	24%	18	8	17.4%
Ma'an					
Ram	30.3.2010	-	15	9	19.6%
Al mudawwara	1.4.2010	20%	10	4	19.6%
	14.4.2010	28%	15	5	
Total			145	46	100%

4.4. Identification and characterization of the causal Agent:

The results of the reaction of the obtained bacterial isolates (isolated from plants suspected to be infected with blackleg) against the different biochemical, physiological and nutritional tests are presented in the Table (2). All the tested isolates were found to be catalase positive, oxidase negative, induce rotting on the inoculated potato slices, oxidative-fermentative positive, hydrolyse starch and negative grow at 37 ºC, positive growth at 5% NaCl, produce nitrite from nitrate, urease negative, H_2S was produced in the test tubes containing cystine broth, Indole was not produced from tryptophane. All isolates produced acid from α-methyl-d-glucoside, reducing substances from sucrose and have induced hypersensitive reaction on tobacco leaves (white burley) injected with the suspension of the tested bacterial isolates (Fig 3-16). The reactions of the tested bacterial isolates to the different biochemical, physiological and nutritional tests were identical to the reactions of the reference culture of *Erwinia carotovora* subsp. *atroseptica* (ATCC 15713) to the same tests.

Table (2). Results of laboratory tests for 46 different *Erwinia carotovora* subsp. *atroseptica* isolates collected from Jordan compare to (ATCC 15713) reference culture.

Isolate	Acid production from α-methyl-d-	Catalase	Growth at 37°C	H_2S production	Hypersensitive reaction to tobacco	Indole production	Nitrate reduction	Oxidase	Oxidative	Potato soft rot	Reducing substances from sucrose	Sodium chloride tolerance 5%	Starch hydrolysis	Urease production
AlKarama & Ash shuna janubiyya (Ka29 and Ka30).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Muthallath al misri (J1, J3, J4, J5, J6, J7, J8, J9 and J10).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Ram (Rm 71, Rm 72, Rm 74, Rm 75, Rm76, Rm 77, Rm 78, Rm 79 and Rm 80).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Kurayyima (Kr26, Kr27 and Kr28).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Tawahin Al udwan (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Dayr Alla (D15, D16, D20 and D23).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Dirar (Rb24 and Rb25)	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Al mudawwara (April.14) (MM33, MM34, MM35, MM37 and MM41).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Al mudawwara (April.1) (MA85, MA86, MA88 and MA89).	+	+	-	+	+	-	+	-	+	+	+	+	-	-
Reference culture *Eca* (ATCC 15713).	+	+	-	+	+	-	+	-	+	+	+	+	-	-

Ref*: Reference culture *Eca* (ATCC 15713)

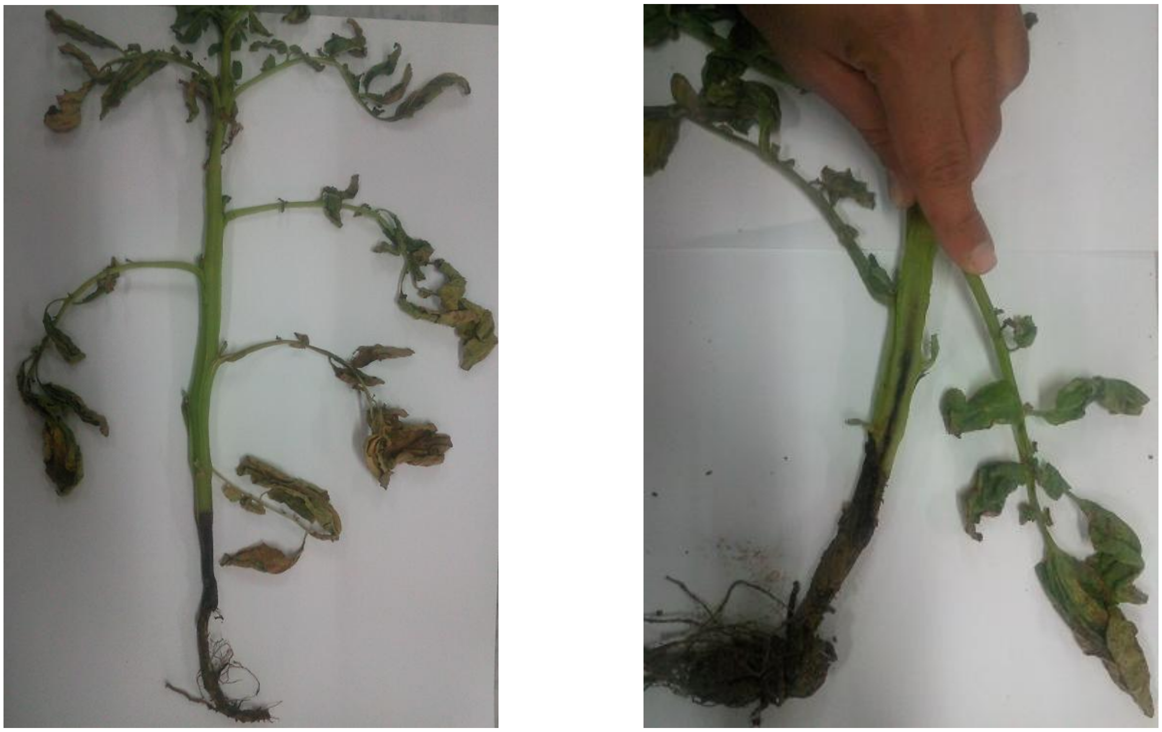


Fig. (1) Potato stems infected with blackleg.

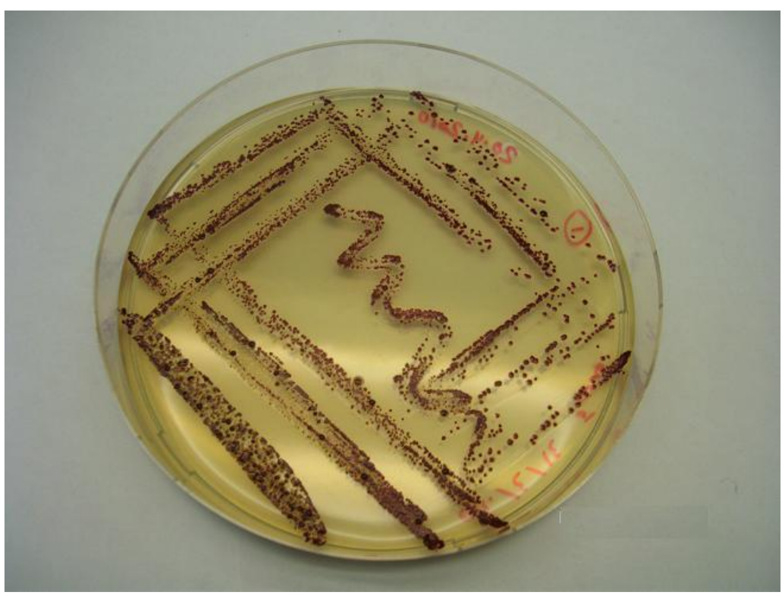
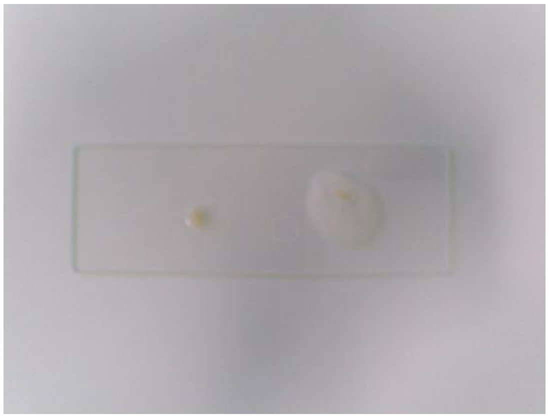


Fig. (2): Growth of *Erwinia carotovora* subsp. *atrosepica* on Logan's medium.



A B

Fig. (3), Catalase test,

(A) Negative reaction, *Enterococcus faecalis.*

(B) Positive reaction, *Eca.*



A B

Fig. (4), Oxidase test.

(A) Positive reaction, *Pseudomonas aeruginosa.*

(B) Negative reaction, *Eca.*

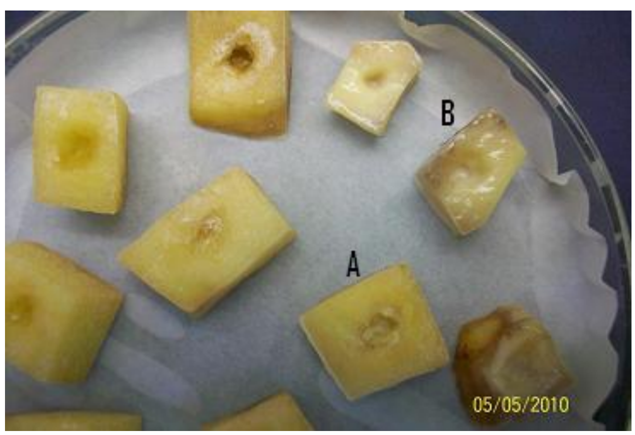


Fig. (5), Potato soft rot test.

(A) Negative reaction, *Pseudomonas syringae* pv. *tomato*

(B) Positive reaction, *Eca.*

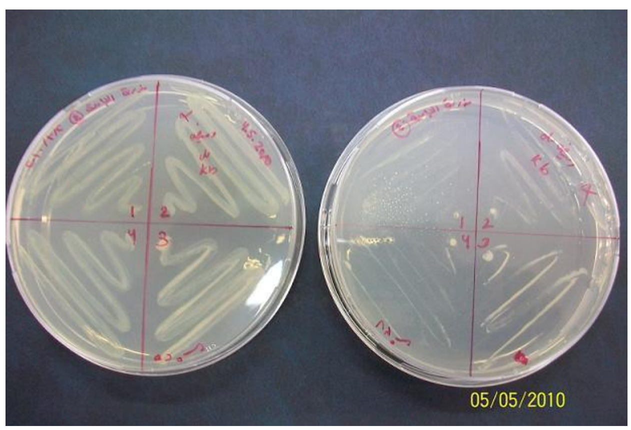


A B

Fig. (6), Oxidative – fermentative test,

(A): Negative reaction, *Pseudomonas syringae* pv. *tomato*

(B): Positive reaction, *Eca.*



A B

Fig. (7), Growth at 37°C,

(A) Positive growth, *Ecc.*

(B) Negative growth, *Eca.*



A B

Fig. (8), Sodium chloride tolerance

(A) Positive growth, *Eca.*

(B) Negative growth, *Ralstonia solanacearum*

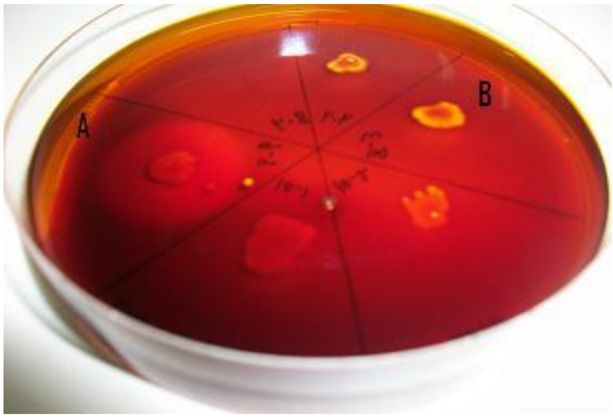


Fig. (9), Starch hydrolysis test

(A) Positive reaction, *Xanthomonas campestris* pv. *phaseoli*

(B) Negative reaction, *Eca.*



A B

Fig. (10), Nitrate reduction test,

(A) Negative reaction, *Erwinia amylovora*

(B) Positive reaction, *Eca*.

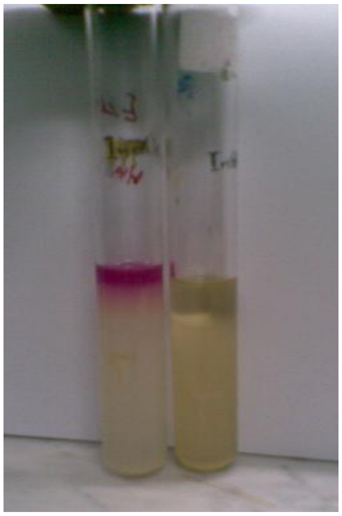


A B

Fig. (11), H_2S production test,

(A) Negative reaction, *Erwinia amylovora*

(B) Positive reaction, *Eca*.



A B

Fig. (12), Indole production test

(A) Positive reaction, *Erwinia chrysanthemi*

(B) Negative reaction, *Eca.*

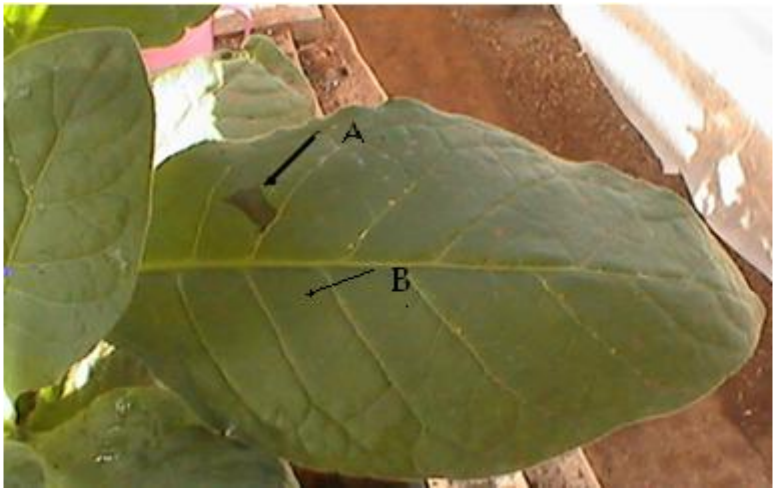
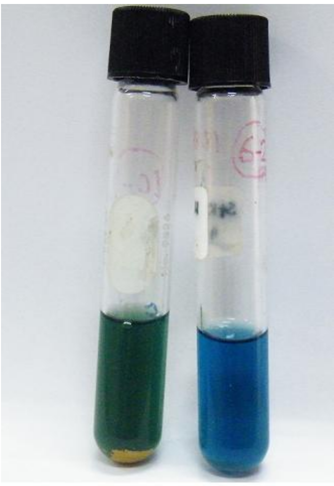


Fig. (13). Hypersensitive reaction (HR) to white burley tobacco leaves.

(A) Positive reaction, *Eca.*

(B) Negative reaction, *Xanthomonas campestris* pv. *phaseoli*



A B

Fig. (14), Reducing substances from sucrose.

(A) Positive reaction, *Eca.*

(B) Negative reaction, *Ecc*



A B

Fig. (15), Urease production test.

(A) Positive reaction, *Ralstonia solanacearum*

(B) Negative reaction, *Eca.*

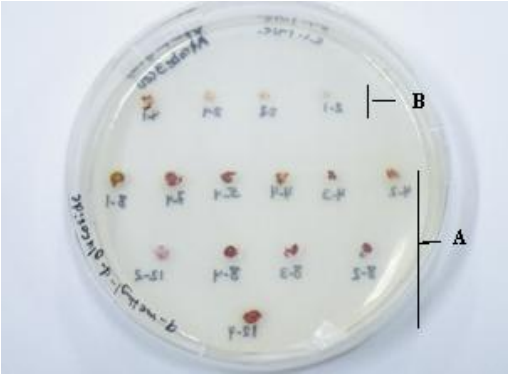


Fig. (16), Acid production from α-methyl-d-glucoside

(A) Positive reaction, *Eca.*

(B) Negative reaction, *Ecc*

4.5. Detection of the 690-bp band from bacterial cultures:

The DNA Extract of the 46 *Eca* isolates amplified with PCR produced the 690-bp band using ECA 1f and ECA 2r primers (Fig. 17-24).

4.6. PCR-RFLP and cluster analysis:

Digestion of the PCR amplification products with restriction enzymes *Sau*3*A*I, *Hpa*II, *Hae*III, and *Alu*I for the tested bacterial isolates formed different fragment patterns for the 690-bp bands Appendix (1). Digestion with *Sau3A*I gave fragment sizes of 447, 126, 71 and 50-bp, HpaII gave fragment sizes of 251, 282, 316, 355, 374 and 398-bp, *Hae*III gave fragment sizes of 126, 158, 199, 224 and 478-bp, while there was no digestion using *Alu*I enzyme (Fig 25-31).

Based on the genetic distances, isolates were clustered in five different groups as shown in Figure 32. The first group included only one isolate (Rm80) where the digestion of the amplified PCR products of the 690-bp band resulted in bands formation of (447, 126, 71 and 50-bp); (251 and 374-bp); and (199 and 478-bp) using *Sau*3*A*I, *Hpa*II and *Hae*III enzymes respectively (Fig. 29). In the other hand, the second group included the isolates (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66), these isolates produced similar bands (447, 126, 71 and 50-bp), (282 and 398-bp), and (126, 158 and 224-bp) using *Sau*3*A*I, *Hpa*II and *Hae*III restriction enzymes, respectively.

Isolates J1, J3, J4, J5, J6, J7, J8, J9, and J10 were clustered in group number 3. These isolates produced similar bands (447, 126, 71 and 50-bp); (282 and 316-bp); and (126,158 and 224-bp) using *Sau*3*A*I, *Hpa*II and *Hae*III restriction enzymes, respectively. Group number 4 included the isolates Rm71, Rm72, Rm74, Rm75, Rm76, Rm77, Rm78, Rm79, Rb24, Rb25, Kr26, Kr27, kr28, MA85, MA86, MA88, MA89, MM33, MM34, MM35, MM37, and MM41. All these 22 isolates produced the digested bands (447, 126, 71 and 50-bp); (251 and 374-bp);

and (126, 158 and 224-bp), resulted from using *Sau*3*A*I, *Hpa*II and *Hae*III restriction enzymes, respectively.

Finally, the isolates (Ka29, Ka30, D15, D16, D20 and D23) were fallen in the fifth group. The PCR amplified product of the 690-bp of these isolates resulted in bands formation of (447, 126, 71 and 50-bp) (251 and 355-bp); and (126,158 and 224-bp); when this region was digested with *Sau*3*A*I, *Hpa*II and *Hae*III restriction enzymes, respectively.

The genetic similarity between the 46 *Erwinia carotovora* subsp *atroseptica* isolates ranged from 1.00 between isolates within each group to 0.471 between group 1 and groups (2 & 3). On average, group 1 showed a genetic similarity of 0.60 with all other groups, is showed a genetic similarity of 0.706 and 0.589 with group 4 and 5, respectively. Equal genetic similarity of 0.889 was observed between group 2 and 3, and between group 4 and 5. The genetic distance between groups (2 & 3) and groups (4 & 5) was 0.778.

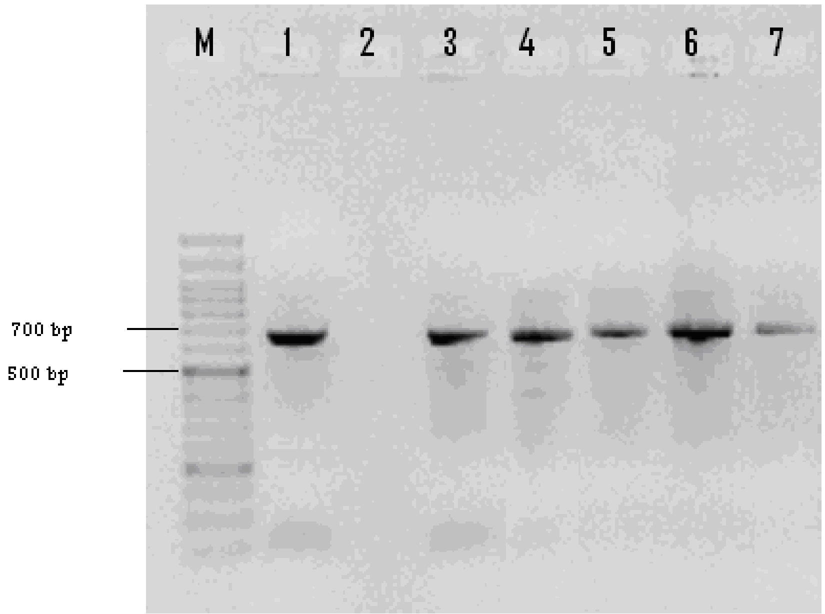


Fig (17): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica*, Lane 2 water control, Lane 3 and 4 for (Rb24, Rb25), Lane 5-7 for (Kr26, Kr27 and Kr28).

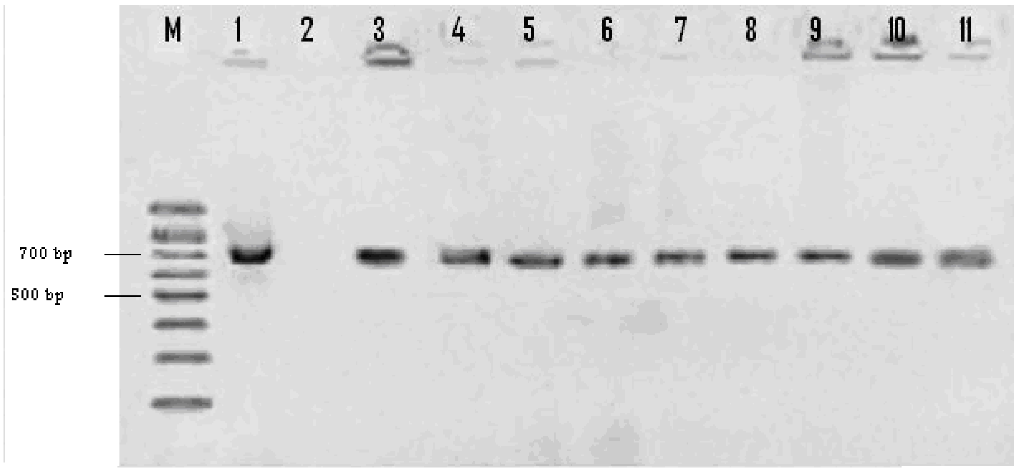


Fig (18): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica*, Lane 2 water control, Lane 3-11 for (Rm71, Rm72, Rm74, Rm75, Rm76, Rm77, Rm78, Rm79 and Rm80).

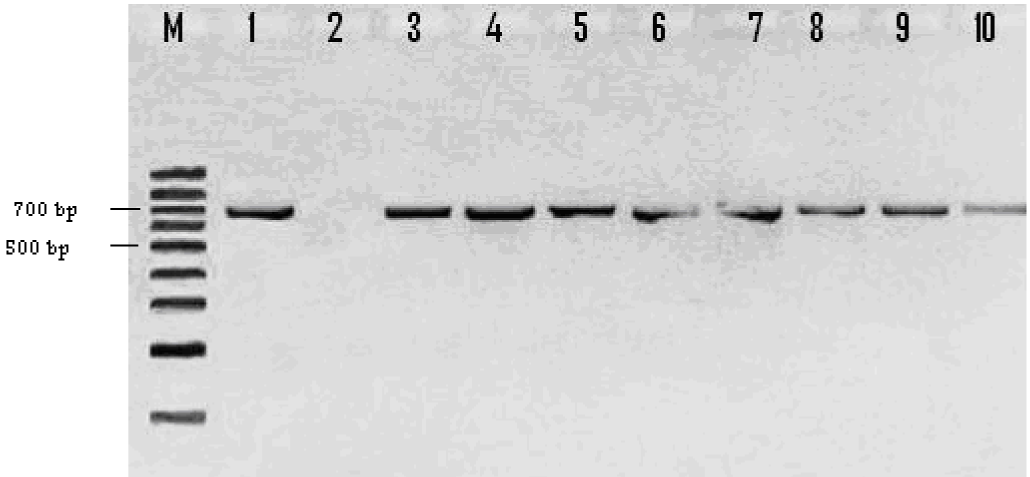


Fig (19): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica*, Lane 2 water control, Lane 3-10 for (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66).

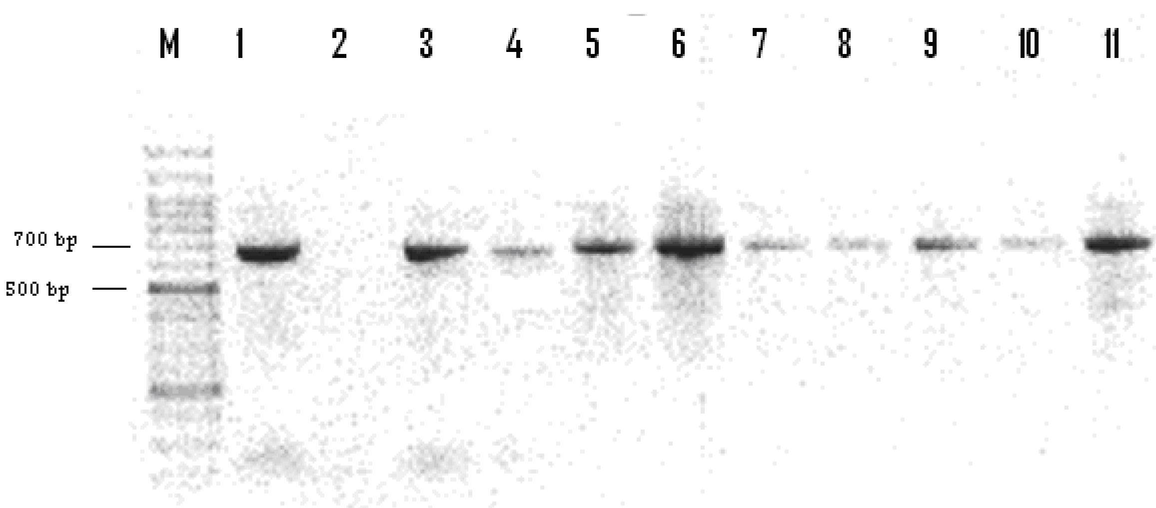


Fig (20): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica*, Lane 2 water control, Lane 3-11 for (J1, J3, J4, J5, J6, J7, J8, J9 and J10).

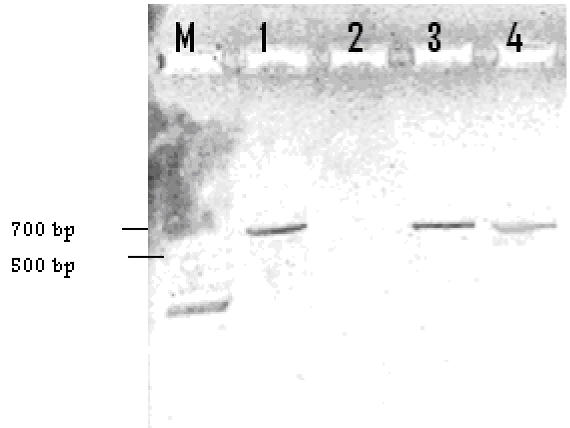


Fig (21): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica,* Lane 2 water control, Lane 3 and 4 for (Ka29 and Ka30).

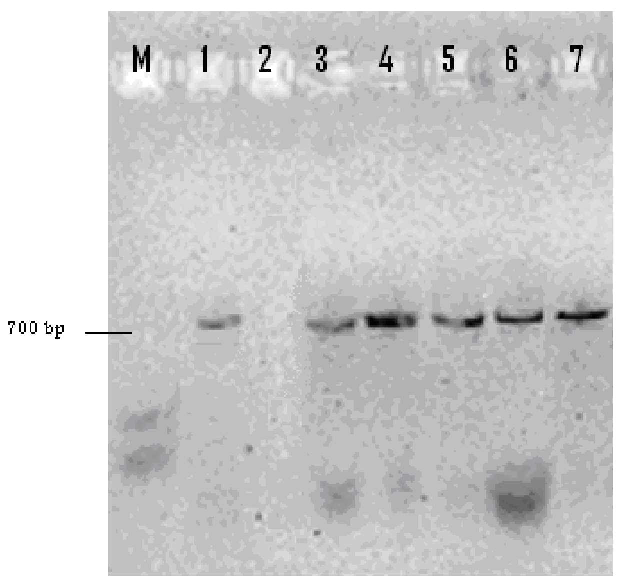


Fig (22): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane M, 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica,* Lane 2 water control, Lane 3-7 for (MM33, MM34, MM35, MM37 and MM41).

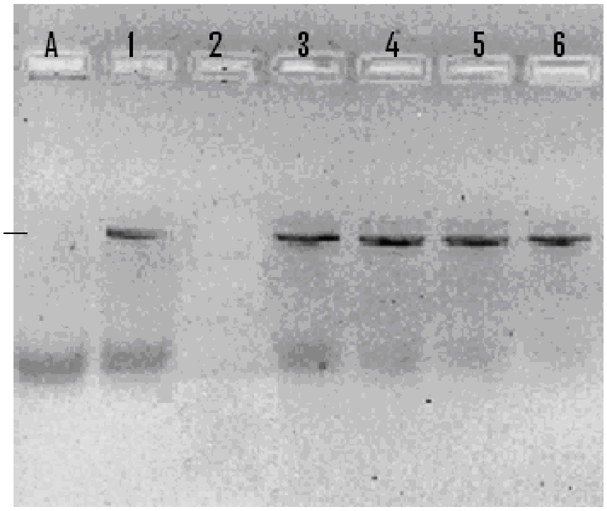


Fig (23): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica,* Lane 2 water control, Lane 3-6 for (MA85, MA86, MA88 and MA89).

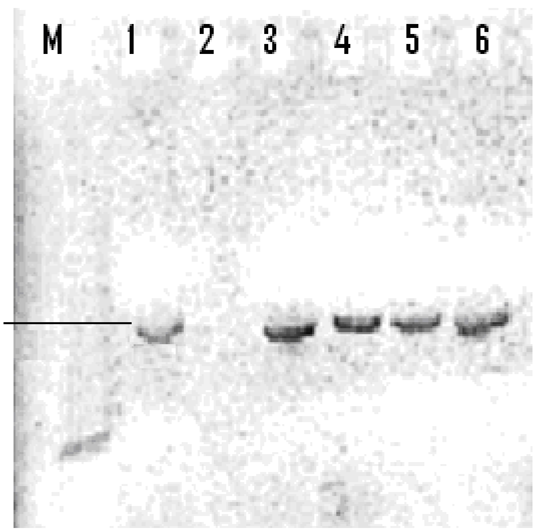


Fig (24): PCR-amplified *Eca* DNA (690-bp band) detection by gel electrophoresis: Lane (M), 100-bp molecular size marker, lane 1, Positive control using DNA extracted from bacterial culture of reference *Erwinia carotovora* subsp. *atroseptica,* Lane 2 water control, Lane 3-6 for (D15, D16, D20 and D23).

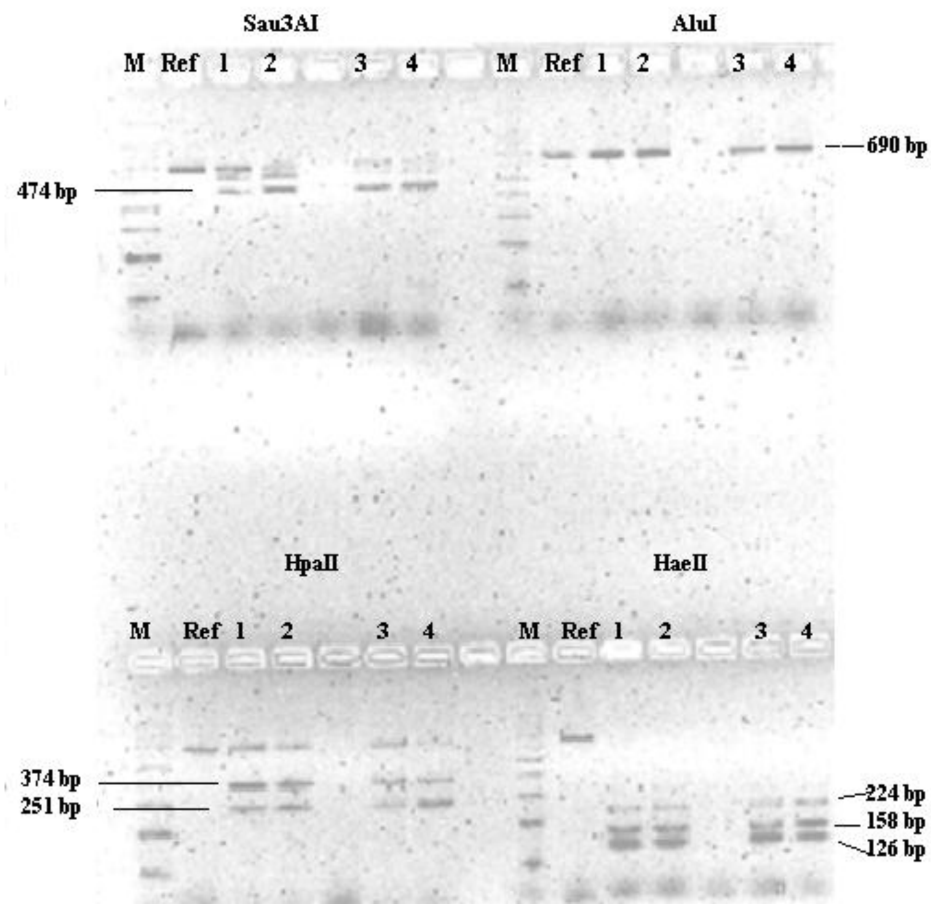


Fig. (25): Restriction pattern of Almudawwara (April, 1) *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, HpaII and HaeIII) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR products (690-bp), Lane 1- 4 for (MA85, MA86, MA88, and MA89) digested by *Sau*3AI, *Alu*I, *Hpa*II and *Hae*III enzymes.

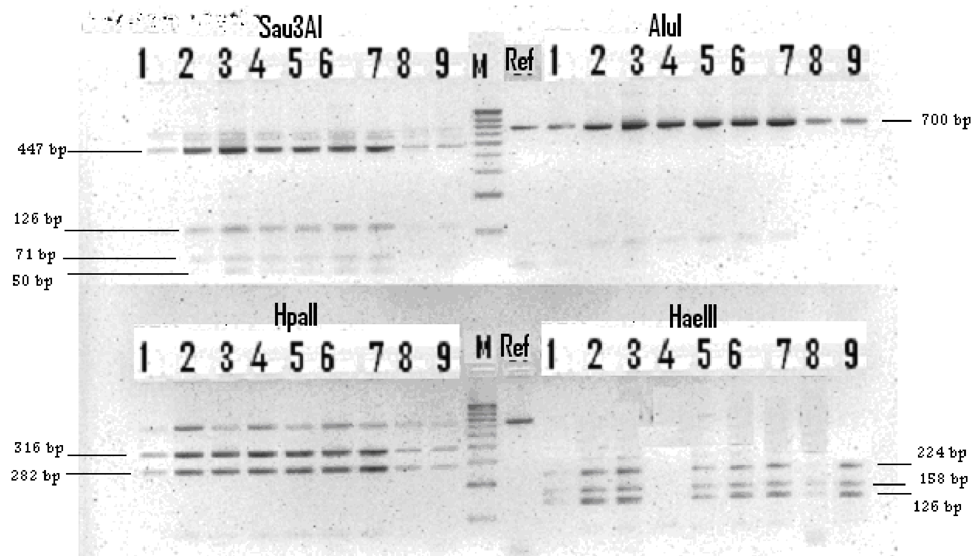


Fig. (26): Restriction pattern of Muthallath al misri *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II and *Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR product (690-bp), Lane 1-9 for (J1, J3, J4, J5, J6, J7, J8, J9 and J10) digested by *Sau*3AI, *Alu*I, *Hpa*II and *Hae*III enzymes.

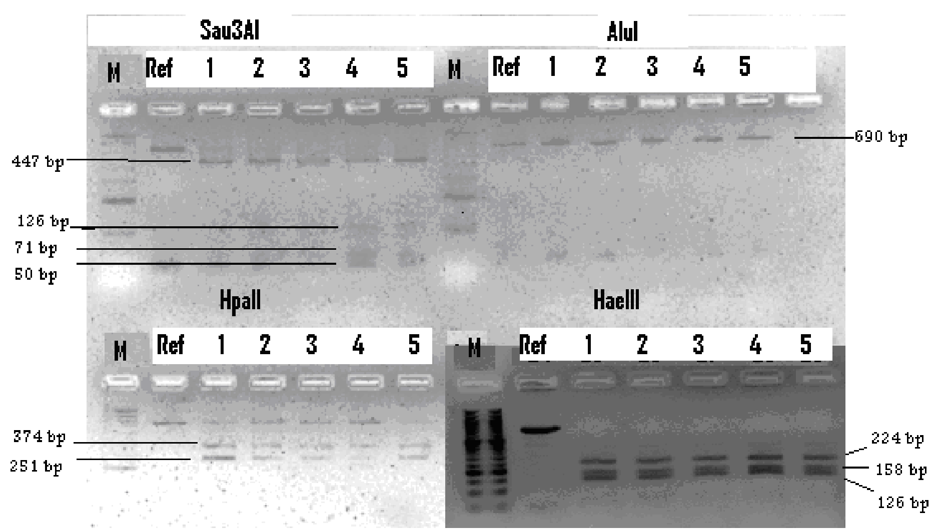


Fig. (27): Restriction pattern of Al mudawwara (April, 14) *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II and *Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR product (690-bp), Lane 1-5 for (MM33, MM34, MM35, MM37, MM41) digested by with (*Sau*3AI, *Alu*I, *Hpa*II and *Hae*III) enzymes.

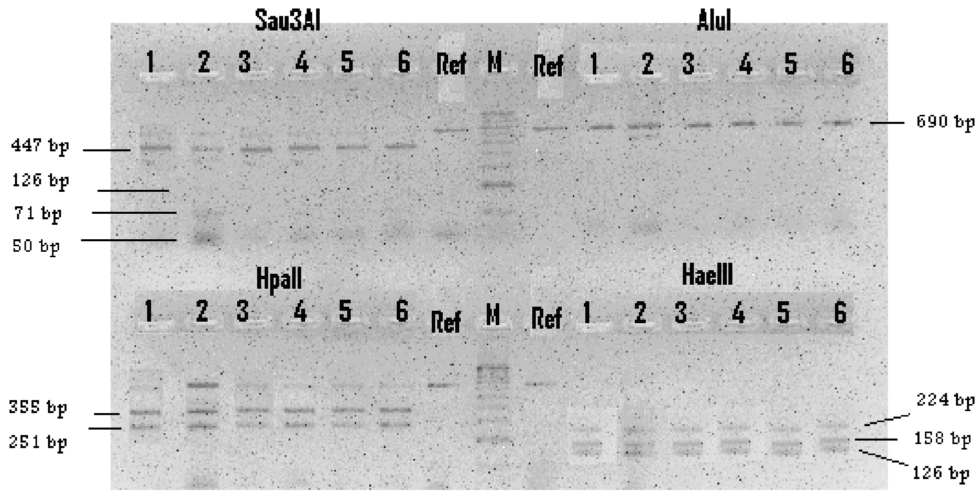


Fig. (28): Restriction pattern of Dayr Alla and AlKarama, Ash shuna janubiyya *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II and *Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR product (690-bp), Lane 1- 6 for (Ka29, Ka30, D15, D16, D20 and D23) digested by *Sau*3AI, *Alu*I, *Hpa*II and *Hae*III enzymes.

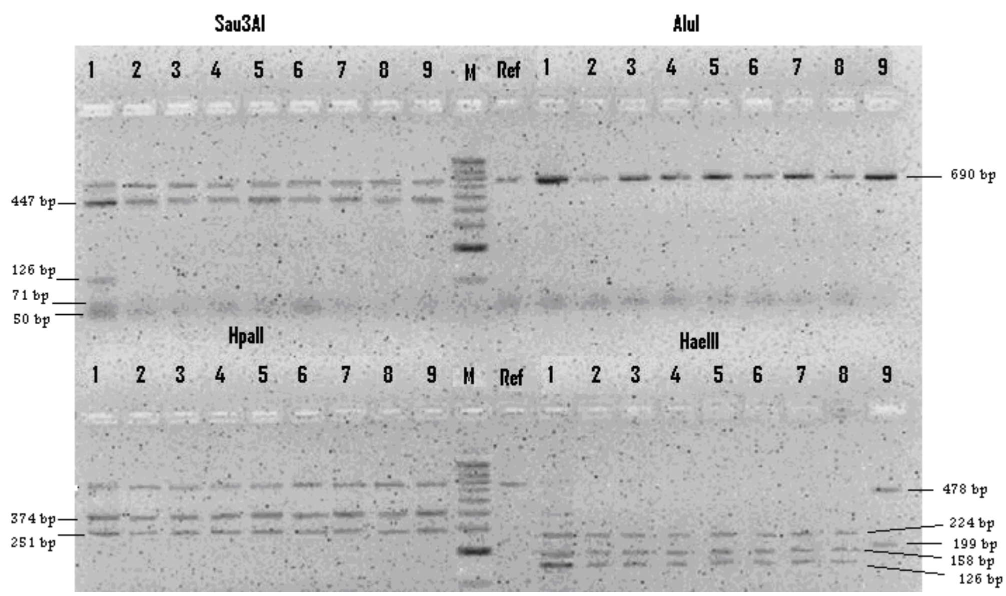


Fig. (29): Restriction pattern of Ram *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II *and Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR products (690-bp), Lane 1- 9 for (Rm 71, Rm 72, Rm 74, Rm 75, Rm76, Rm 77, Rm 78, Rm 79 and Rm 80) digested by *Sau*3AI, *Alu*I, *Hpa*II and *Hae*III enzymes.

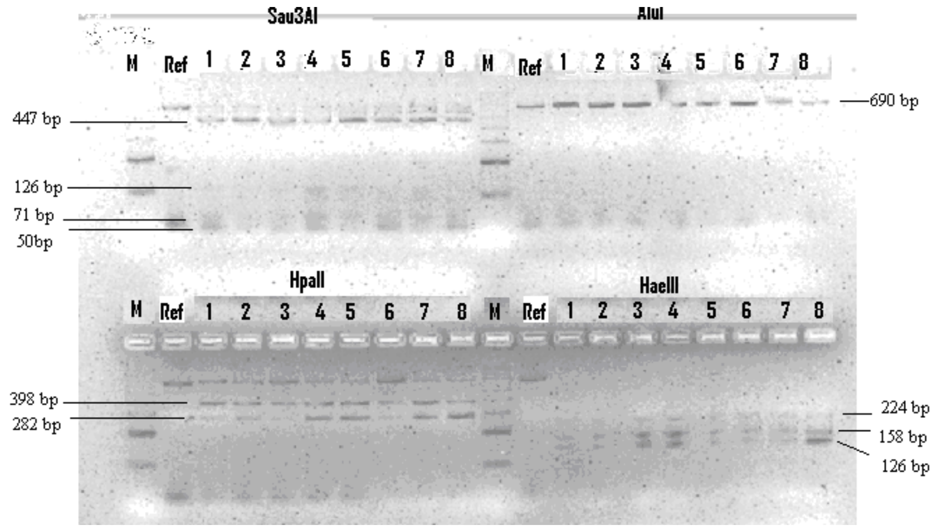


Fig. (30): Restriction pattern of Tawahin Al udwan *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II *and Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR products (690-bp), Lane 3- 10 for (Ta59, Ta60, Ta61, Ta62, Ta63, Ta64, Ta65 and Ta66) digested by *Sau*3AI, *Alu*I, *Hpa*II *and Hae*III enzymes.

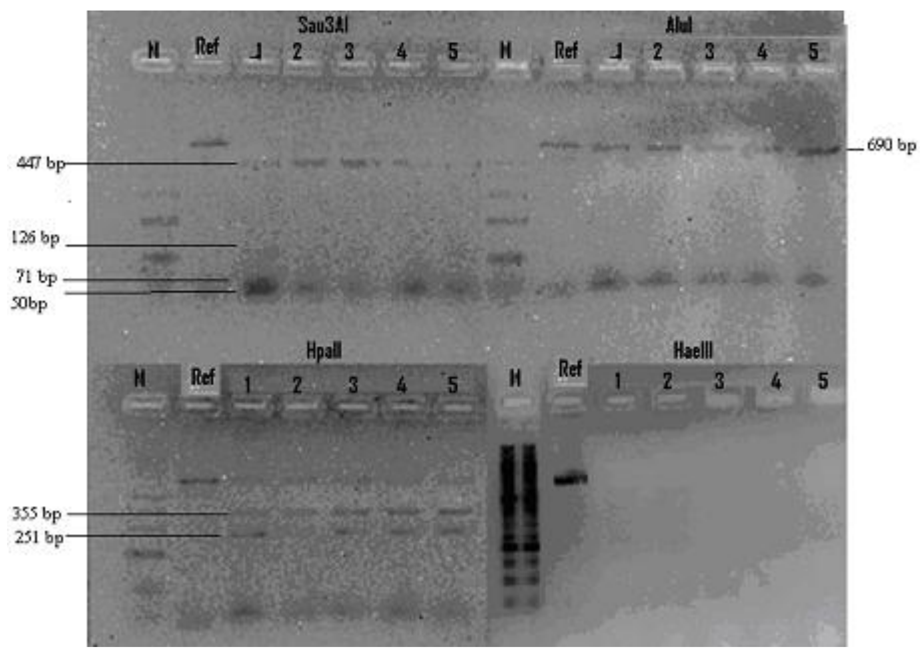


Fig. (31): Restriction pattern of Dirar and Kurayyima *Eca* isolates for 690-bp band after digestion with (*Sau*3AI, *Alu*I, *Hpa*II and *Hae*III) enzymes. Lane M, 100-bp molecular size marker, Lane Ref, uncut PCR products (690-bp), Lane 1- 5 for (Rb24, Rb25, Kr26, Kr27, and Kr28) digested by *Sau*3AI, *Alu*I, *Hpa*II and *Hae*III enzymes.

Table (3): Distance matrix of *Erwinia carotovora* subsp *atroseptica* based on PCR-RFLP of the 690-bp amplified fragments.

isolate	**Ka29**	**Ka30**	**D15**	**D16**	**D20**	**D23**	**Rm71**	**Rm72**	**Rm74**	**Rm75**	**Rm76**	**Rm77**	**Rm78**	**Rm79**	Rm80	Rb24	Rb25	Kr26	Kr27	Kr28	MA85	MA86	MA88	MA89
Ka29	1																							
Ka30	1	1																						
D15	1	1	1																					
D16	1	1	1	1																				
D20	1	1	1	1	1																			
D23	1	1	1	1	1	1																		
Rm71	0.889	0.889	0.889	0.889	0.889	0.889	1																	
Rm72	0.889	0.889	0.889	0.889	0.889	0.889	1	1																
Rm74	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1															
Rm75	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1														
Rm76	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1													
Rm77	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1												
Rm78	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1											
Rm79	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1										
Rm80	0.589	0.589	0.589	0.589	0.589	0.589	0.706	0.706	0.706	0.706	0.706	0.706	0.706	0.706	1									
Rb24	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1								
Rb25	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1							
Kr26	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1						
Kr27	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1					
Kr28	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1				
MA85	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1			
MA86	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1		
MA88	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	
MA89	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
MM33	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
MM34	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
MM35	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
MM37	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
MM41	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1	0.706	1	1	1	1	1	1	1	1	1
J1	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J3	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J4	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J5	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J6	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J7	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J8	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J9	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
J10	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta59	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta60	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta61	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta62	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta63	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta64	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta65	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778
Ta66	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.471	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778	0.778

Continued Table (3): Distance matrix of *Erwinia carotovora* subsp *atroseptica* based on PCR-RFLP of the 690-bp amplified fragments the 690-bp amplified fragments.

isolate	MM33	MM34	MM35	MM37	MM41	J1	J3	J4	J5	J6	J7	J8	J9	J10	Ta59	Ta60	Ta61	Ta62	Ta63	Ta64	Ta65	Ta66
Ka29																						
Ka30																						
D15																						
D16																						
D20																						
D23																						
Rm71																						
Rm72																						
Rm74																						
Rm75																						
Rm76																						
Rm77																						
Rm78																						
Rm79																						
Rm80																						
Rb24																						
Rb25																						
Kr26																						
Kr27																						
Kr28																						
MA85																						
MA86																						
MA88																						
MA89																						
MM33	1																					
MM34	1	1																				
MM35	1	1	1																			
MM37	1	1	1	1																		
MM41	1	1	1	1	1																	
J1	0.778	0.778	0.778	0.778	0.778	1																
J3	0.778	0.778	0.778	0.778	0.778	1	1															
J4	0.778	0.778	0.778	0.778	0.778	1	1	1														
J5	0.778	0.778	0.778	0.778	0.778	1	1	1	1													
J6	0.778	0.778	0.778	0.778	0.778	1	1	1	1	1												
J7	0.778	0.778	0.778	0.778	0.778	1	1	1	1	1	1											
J8	0.778	0.778	0.778	0.778	0.778	1	1	1	1	1	1	1										
J9	0.778	0.778	0.778	0.778	0.778	1	1	1	1	1	1	1	1									
J10	0.778	0.778	0.778	0.778	0.778	1	1	1	1	1	1	1	1	1								
Ta59	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1							
Ta60	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889		1						
Ta61	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1					
Ta62	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1				
Ta63	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1			
Ta64	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1		
Ta65	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	
Ta66	0.778	0.778	0.778	0.778	0.778	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	0.889	1	1	1	1	1	1	1	1

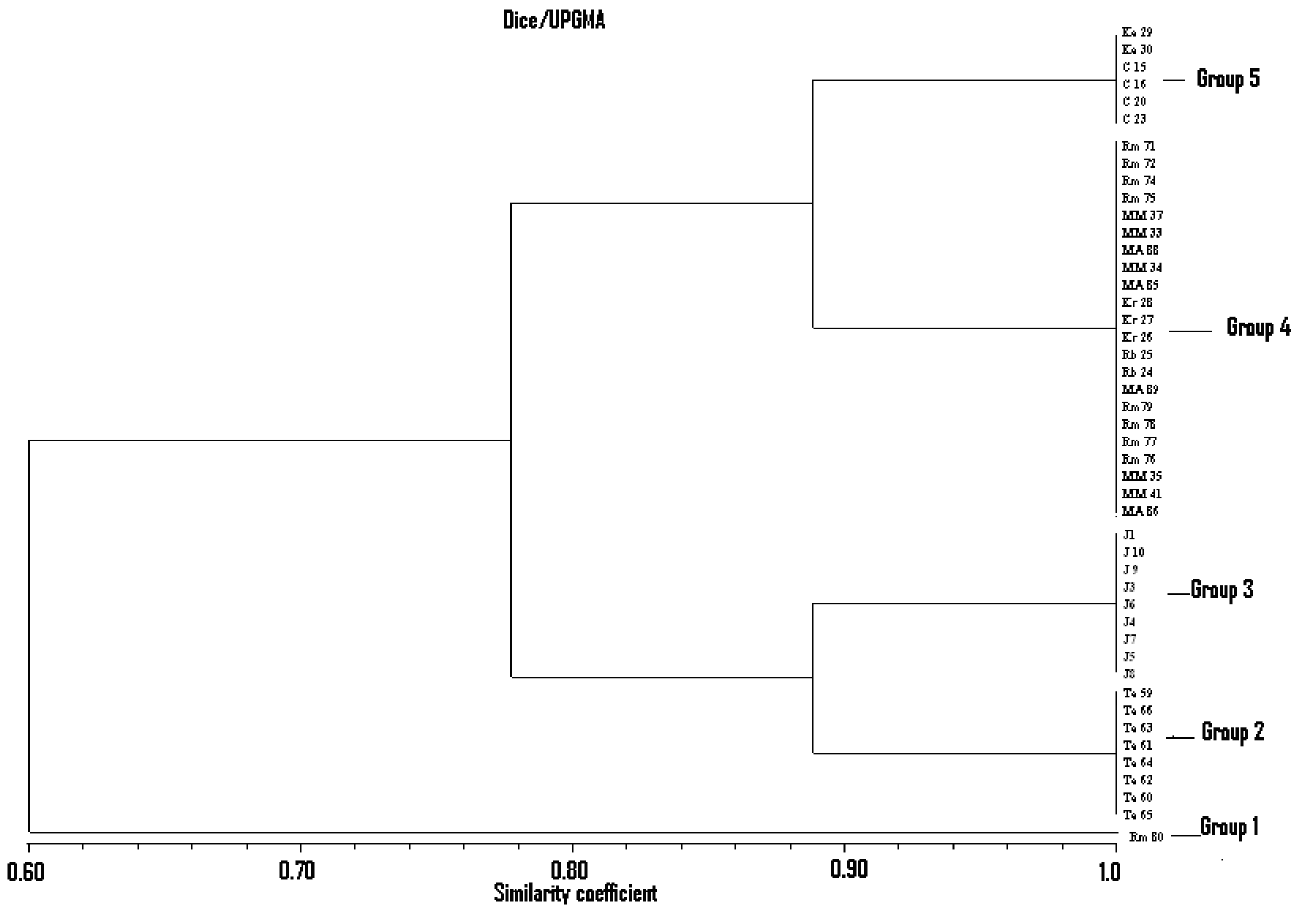


Fig. (32): Dendogram showing genetic relationships between the 46 *Erwinia carotovora* subsp. *atroseptica* isolates at chromosomal level based on PCR-RFLP analysis of the 690-bp band. The tree was constructed by using the UPGMA method with table 3 data.

4.7. Detection of *Eca* from naturally infected potato plant tissues:

The results of direct isolation from naturally infected potato tubers and stem samples on Logan's media and identification as previously mentioned are presented in (Table 4). The results show that the pathogen *Eca* was isolated from 30% of the tested samples when their suspension was streaked on Logan's media plates. While the 690-bp band was detected in 88% of the DNA extracted from the same diseased samples amplified with PCR and horizontally separated by gel electrophoreses (Fig. 33).

4.8. Detection of *Eca* from symptomless potato plant and tuber samples:

The results of direct isolation identified from symptomless potato tubers and stem samples on Logan's media and identification as previously mentioned are presented in (Table 4 and 5). The results showed that *Eca* was not recovered from the symptomless potato stem samples, when their suspension was streaked on Logan's media plates. While the 690-bp was detected in 8 % of the DNA extracted from the same symptomless potato stem samples amplified with PCR and horizontally separated by gel electrophoreses.

The results showed that *Eca* was recovered only in 4% of the symptomless potato tuber samples when their suspension was streaked on Logan's media plates, while the 690-bp was detected in 32% of the DNA extracted from the same symptomless potato tuber samples amplified with PCR and horizontally separated by gel electrophoreses (Fig.34).

Table (4): Direct isolation on Logan's media versus detection of the 690-bp band with PCR from blackleg naturally infected potato plant tissue samples.

Method	Number of tested samples.	Number of samples gave positive result.	Percentage
PCR	50	44	88%
Direct isolation on Logan's media	50	15	30%

Table (5): Direct isolation on Logan's media versus detection of the 690-bp band with PCR from symptomless potato stem samples.

Method	Number of tested samples.	Number of samples gave positive result.	Percentage
PCR	25	2	8%
Direct isolation on Logan's media	25	0	0

Table (6): Direct isolation on Logan's media versus detection of the 690-bp band with PCR from symptomless potato tuber samples.

Method	Number of tested samples.	Number of samples gave positive result.	Percentage
PCR	25	8	36%
Direct isolation on Logan's media	25	1	4%

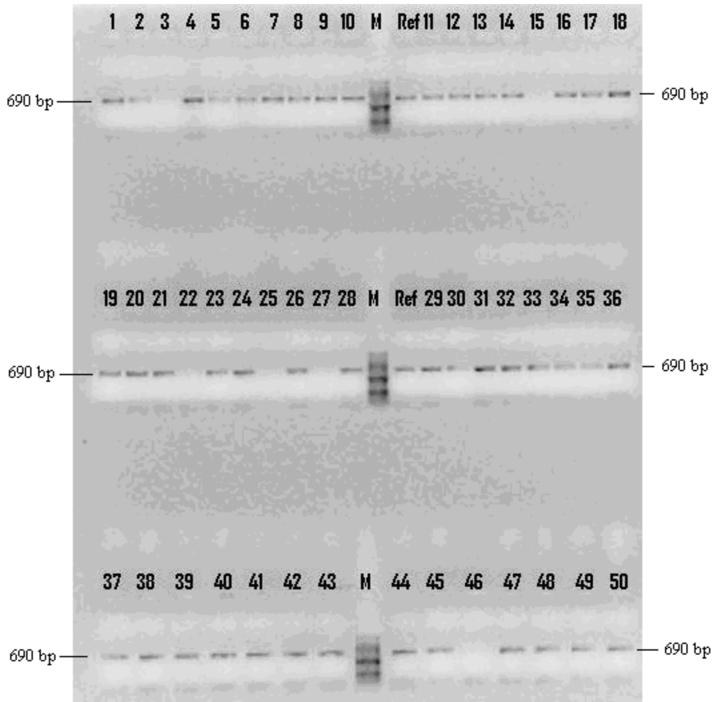


Fig (33): PCR detection of the 690-bp band from plant samples with symptoms: Lane M, 100-bp molecular size marker, Ref, DNA extracted from reference culture, Lane 1-50, naturally infected potato plant tissue samples.

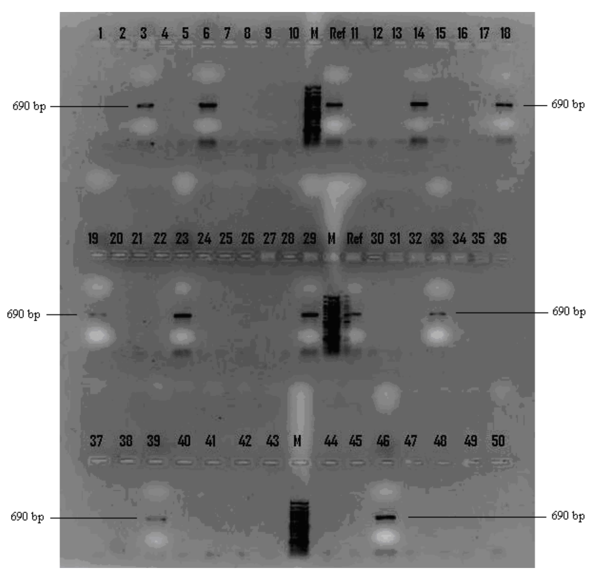


Fig (34): PCR detection of the 690-bp band from plant samples with symptomless: Lane M, 100-bp molecular size marker, Ref, DNA extracted from reference culture, Lane 1-50, symptomless potato tuber and plant samples.

5. Discussion

Field trips to potato growing areas revealed that blackleg disease was found to spread in all surveyed potato growing areas including Jordan Valley (AlKarama & Ash shuna janubiyya, Muthallath al misri, Dayr Alla, Dirar, and Kurayyima), Jarash (Tawahin Al udwan), Ma'an (Ram, and Al mudawwara). The recorded disease incidence during sample collection ranged from 5% up to 28% for Madaba and Al mudawwara (April.14.2010) locations respectively.

Different sub species of *Erwinia* reported to cause blackleg like symptom of these *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Ech*) and *E. carotovora* subsp. *atroseptica* (Degefu *et al.*, 2006).

The reaction of the 46 obtained bacterial isolates isolated from diseased potato samples collected from the different location to the biochemical, physiological and nutritional tests were found similar and identical to the reaction of the reference culture of *Erwinia carotovora* subsp. *atroseptica* (ATCC 15713) to the same tests (Schaad *et al*., 2001, Yahiaoui-Zaidi *et al.*, 2003). This confirmed that *Erwinia carotovora* subsp. *atroseptica* is the causal agent of potato black leg in Jordan (Al-Masa'deh and Khlaif, 2003).

In this study, all the 46 bacterial isolates (*Eca*) produced of 690-bp the expected band in PCR amplification for the DNA extracted from the tested isolates. This result was in agreement with De Boer and Ward (1995) who used ECA1f (5'CGGCATCAT AAAAACACG-3') and ECA2r (5'GCACACTTCATCCAGCGA-3') that specifically amplified a 690-bp DNA fragment of *E. carotovora* subsp. *atroseptica*, the causal agent of potato blackleg, but not other *E. carotovora* subspecies.

Our results showed that *Eca* was isolated on Logan's media from 30% of naturally infected potato plant tissue when their suspensions were streaked on Logan's media, in the same time 88% of the DNA extracted from these samples produced the 690-bp amplification by PCR.

While in the case of symptomless potato tubers and stem samples, *Eca* was not recovered from the suspension of stem samples when their suspensions were streaked on the surface of Logan's media plates, and the 690-bp band was detected in 8% in their DNA extract amplified with PCR, and *Eca* was recovered in 4% of the symptomless potato tuber samples when their suspension were streaked on the surface of Logan's media plates, while the 690-bp was detected in 32% in the DNA extracted from these samples and amplified by PCR.

These results agreement with Frechon *et al*., 1998 who find that PCR (88%) the most sensitive method to detect *Eca* followed by enrichment ELISA (72%) and at last ELISA, the least sensitive method (30%).

Using PCR was found to be more efficient in detecting *Eca* in comparison to isolation on Logan’s medium and identification by biochemical, physiological and nutritional tests.

The efficiency of PCR in detection 690-bp band could be related to that; 690-bp region could be easily detected from plant material without isolation from the expected healthy and infected plant parts within a short time, and the bacterial DNA sequence revealing for *Eca* can be amplified from viable, non-culturable, transformed plant cell. However because *Eca* has a latent infection PCR could be a beneficial tool for detecting latent infection of *Eca* especially in imported potato tuber seeds before distributing to the farmers.

Direct isolation results are in agreement with those of Toth *et al*., (2001) who reported that visual disease symptoms are not always sufficient to make an unequivocal identification of

the pathogen latent infection. Thus, to ensure that a correct diagnosis is made and that steps are taken toward reducing disease spread, identification systems are required. This can be achieved by using detection systems that identify the pathogen directly from plant samples like PCR technique.

The results of PCR-RFLP of the 690-bp digestion band with (*Sau*3AI, *Alu*I, *Hpa*II *and* *Hae*III) enzymes, which obtained with ECA1f and ECA2r primers, indicated the occurrence of genetic relationship among the tested *Eca* isolates.

The tested isolates from the same collected region were found to be identical, since the genetic distance between them was found to be (0.0) as the same banding patterns were formed when their 690-bp bands were digested with the four different restriction enzymes. These results are in agreement with Yahiaoui-Zaidi *et al*., (2003), where molecular fingerprints showed low genetic variability within *Eca* strains using PCR-RFLP after analysis of the restriction patterns of the PCR products obtained with the Y1(5’-TTACCGGACGCCGAG CTGTGGCGT-3’) and Y2 (5’-CAGGAAGATGTCGTTATCGCGAGT-3’) primers. This result could reflect the adaptation of *Eca* to local environmental conditions (Yahiaoui-Zaidi *et al*., 2003).

Our result of RFLP agreed with the results of De Boer & McNaughton, (1987) who showed that 13 strains from *Eca* belong to only two specific PCR-RFLP groups. And the low variability in this subspecies is in agreement with serological studies that indicated the presence of only a few serogroups (De Boer *et al*., 1979).

Moreover, Costa *et al.*, (2006) found low molecular variability between 10 tested isolates of *Eca* collected from Portuguese fields.

6. Conclusion and Recommendations.

1- Blackleg disease is widely spread in the different potato planting areas in Jordan including: Jordan Valley (AlKarama& Ash shuna janubiyya, Muthallath al misri, Mu'addi, Dayr Alla, Dirar, and Kurayyima), Amman (Al yadoda), Madaba (Omalamad), Jarash (Tawahin Al udwan), Ma'an (Ram, and Al mudawwara).

2- Results of biochemical, physiological and nutritional tests, and PCR amplification confirmed that the causal agent of blackleg disease of potato in Jordan is *Erwinia carotovora* subsp. *atroseptica* (Van Hall) Dye.

3- This study indicates that PCR amplification of 690-bp band derived from bacterial culture, naturally infected potato plant and symptomless tubers and plant stems is a sensitive and specific method for detecting *Erwinia carotovora* subsp. *atroseptica* in potato tubers and plants. PCR was found to be more efficient to detect the pathogen than culture methods followed by biochemical, physiological and nutritional tests.

4- Small genetic diversity among collected isolates from Jordan was found (5 RFLP groups) based on PCR-RFLP.

References:

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Appendix (1): PCR-RFLP matrix for 690-bp bands when digested with *Alu*I, *Hae*III, *Hpa*II and *Sau*AI

	Ka 29	Ka 30	D 15	D 16	D 20	D 23	Rm 71	Rm 72	Rm 74	Rm 75	Rm 76	Rm 77	Rm 78	Rm 79	Rm 80	Rb 24	Rb 25	Kr 26	Kr 27	Kr 28	M A 85	M A 86	M A 88	M A 89	M M 33	M M 34	M M 35	M M 37	M M 41	J 1	J 3	J 4	J 5	J 6	J 7	J 8	J 9	J 10	Ta 59	Ta 60	Ta 61	Ta 62	Ta 63	Ta 64	Ta 65	Ta 66
Sau																																														
447	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
126	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
71	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
50	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Alu																																														
690	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hpa																																														
251	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
282	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
316	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
355	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
374	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
398	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
Hae																																														
126	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
158	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
199	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
224	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
478	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

1: denote presence of band, 0: denote absence of band.

الكشف عن وتعريف مرض الساق السوداء على البطاطا

(*Erwinia carotovora* pv. *atroseptica* (Van Hall, 1902))

بإستخدام طريقة تفاعلات البلمرة المتسلسلة

إعداد

رياض الزمر

المشرف

الأستاذ الدكتور حامد خليف

المشرف المشارك

الدكتور مهند العكش

ملخص

دلت نتائج الدراسة الميدانية لاماكن زراعة البطاطا في الاردن على تواجد مرض اسوداد الساق البكتيري على البطاطا في مناطق زراعة البطاطا المختلفة من الاردن والتي شملت: وادي الأردن (الشونة الجنوبية، الكرامة، دامية، مثلث المصري، دير علا، كريمة ، الشونة الشمالية)، عمان (اليادودة)، مادبا (أم العمد)، جرش (طواحين العدوان)، معان (رم ، المدورة).

تم عزل وتعريف 46 عزلة من البكتيريا *Eca* المسبب المرضي لمرض إسوداد الساق من المناطق المختلفة.

إستخدمت طريقة تفاعل البلمرة المتسلسل (PCR) للكشف عن (690-bp band) في مستخلص ال DNA من معلق ال 46 عزلة التي تم الحصول عليها من النباتات المصابة في تفاعل البلمرة المتسلسل تم كشف وجود ال (690-bp band) في كل العزلات.

وفي دراسة أخرى للمقارنة بين كفاءة العزل المباشر على البيئة الغذائية (Logan's) واستخدام تقنية تفاعل البلمرة المتسلسل للكشف عن (690-bp band). أشارت النتائج انه تم عزل المسبب المرضي من 30% من عينات البطاطا المصابة بينما تم الكشف عن (690-bp band) في 88% من مستخلص ال DNA لنفس

العينات بإستخدام ال PCR. كما تم اجراء الكشف عن وجود المسبب المرضي في عينات نباتات بطاطا لا تظهر عليها أ عراض المرض دلت النتائج انه لم يعزل المسبب المرضي مباشرة على البيئة الغذائية (Logan's). بينما تم كشف ال (690-bp band) في 8% عندما استخدم مستخلص ال DNA من هذه العينات في تفاعل ال PCR. عند اجراء الكشف عن وجود المسبب المرضي في درنات بطاطا لا تظهر عليها اعراض الاصابة تم عزل *Eca* من 4% من هذه العينات على البيئة الغذائية (Logan's)، بالمقابل تم الحصول على (690-bp band) من 32% من مستخلص DNA لنفس العينات بإستخدام طريقة ال PCR.

للكشف عن التباين الوراثي بين ال 46 عزلة التي تم جمعه استخدمت طريقة RFLP-PCR

تم هضم ال (690-bp band) بواسطة استخدام الانزيمات *Alu*I, *Hae*III, *Hpa*II and *Sau*AI فقسمت العزلات الى خمس مجموعات ولكن هذه المجموعات ذات تباين وراثي قليل .